INVESTIGATION OF THE BIOSYNTHESIS OF UNSATURATED FATTY ACIDS IN YEAST

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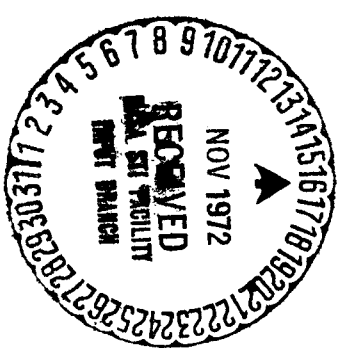

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INVESTIGATION OF THE BIOSYNTHESIS OF UNSATURATED FATTY ACIDS IN YEAST

J. Schultz and F. Lynen[1]

ABSTRACT. 1. Palmitoyl-CoA and other longchain acyl-CoA derivatives were purified by counter current distribution according to Craig or by partition chromatography on Sephadex G-50 columns. The products obtained were of high purity.

2. The particle bound acyl-CoA desaturase from baker's yeast was isolated. This microsomal fraction contained a considerable amount of fatty acid synthetase which was removed from the microsomes by ultrasonic treatment. The enzyme fraction used contained a very active acyl-CoA hydrolase which was characterized by determination of the Michaelis constants for various longchain acyl-CoA derivatives. The K_m's were found to be about 10^{-5} M for C_{12} to C_{18} fatty acids.

3. By incubation of the desaturase with equimolar amounts of palmitoyl-CoA and radioactive CoASH it was shown that a reaction mechanism proposed by A. T. James for desaturation of fatty acids in yeast is not valid. There is no indication that transfer of an acyl residue from CoA to the enzyme as a first step occurs in the reaction sequence. The enzyme reaction is not inhibited by SH-blocking reagents. It is concluded that an AC--protein-like component does not participate in the enzymatic desaturation of fatty acids in yeast.

4. The Michaelis constants for the substrates stearoyl-, palmitoyl- and myristoyl-CoA for the desaturation reaction were determined to be about 2.8 × × 10^{-6} M. The inhibition of the desaturase by palmitoleoyl-CoA and oleoyl-CoA was shown to be competitive and the inhibitor constants were 2 × 10^{-5} M respectively.

5. The enzyme actively converted decanoyl-CoA to a 9-decenoic acid derivative. The appearance of mostly oleic acid and palmitoleic acid in yeast is explained, therefore, not by the specificity of the desaturase, but by the specificity of the fatty acid synthetase which provides no other substrates for the desaturase than palmitoyl-CoA and stearoyl-CoA.

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The biosynthesis of simple unsaturated fatty acids in yeast was studied by /48* Bloch and James et al. [1-3]. The fatty acid desaturase of the yeast was found in the microsomal fraction and requires the longchain acyl-CoA compound as a substrate. Molecular oxygen and NADPH or NADH are required for desaturation. Similar reaction pathways for the biosynthesis of unsaturated fatty acids have been described for *Torulopis utilis*, *Mycobacterium phlëi*, *Euglena gracilis*, spinach chloroplasts and rat liver [4-8].

The dependence of fatty acid desaturation on the presence of molecular oxygen indicates a hydroxylase reaction. Dehydration performed after hydroxylation would therefore lead to the unsaturated fatty acids. The nine- or ten-hydroxy fatty acids which are probable intermediate products could not be detected, however [1-3]. Accordingly, it was found that the various nine- or ten- hydroxy fatty acids can not be converted from desaturase to unsaturated fatty acids. For this reason, James [2] proposed a mechanism for desaturation in which the residual acyl is transferred in an initial stage of the reaction from CoA to the enzyme, while the hydroxy fatty acid which results as an intermediate product remains enzyme-bound and is restored only after dehydration [Gl. (1-4)]. In view of the results of our studies of the synthesis of saturated fatty acids in fatty acid synthetase from yeast cells [9], we feel that it is possible for a component similar to the AC-protein to act as the acyl acceptor group in these desaturases.

$$\text{Palmitoyl-CoA} + \text{Enzyme} \rightleftharpoons \text{Palmitoyl-Enzyme} + \text{CoASH} \quad (1)$$

$$\text{Palmitoyl-Enzyme} \xrightarrow[-H_2O]{O_2,\ NADPH,\ H^+} \text{Hydroxypalmitoyl-Enzyme} \quad (2)$$

$$\text{Hydroxypalmitoyl-Enzyme} \xrightarrow{-H_2O} \text{Palmitoleoyl-Enzyme} \quad (3)$$

$$\text{Palmitoleoyl-Enzyme} + \text{CoASH} \rightleftharpoons \text{Palmitoleoyl-CoA} + \text{Enzyme} \quad (4)$$

$$\text{Palmitoyl-CoA} + [^{14}C]\text{CoASH} \rightleftharpoons \text{Palmitoyl-}[^{14}C]\text{CoA} + \text{CoASH} \quad (5)$$

*Numbers in the margin indicate pagination in the foreign text.

This would explain why the hydroxy acids could not be detected as intermediate products of desaturation and the nine- or ten- hydroxyacyl-CoA compounds were not converted by the enzyme. The reason would be that the hydroxyacyl residue could not be transferred between the CoA and the enzyme. /49

In order to determine whether the conversion from palmitic acid to the desaturase enzyme occurs according to equation (1), the expected exchange reaction was studied with radioactive CoASH [equation (5)]. We also checked to determine whether the activity of the desaturating yeast system can be inhibited by reagents which block the SH groups, as is the case for the desaturase from rat liver [10]. In addition, the Michaelis constants of several longchain acyl-CoA compounds were determined for the desaturase and the acyl-CoA hydrolyase which also is contained in the microsomal fraction.

Experimental Section

Materials and Methods

Commercial Preparations. CoASH, NADPH (Boehringer Mannheim GmbH (Mannheim); [1-^{14}C] capric acid, [1-^{14}C] lauric acid, [1^{-14}C] myristic acid, [1^{-14}C] palmitic acid, [1^{-14}C] stearic acid (Radiochemical Center, Amersham); capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, myristoleic acid, palmitoleic acid, oleic acid (C. Roth, Karlsruhe); iodacetamide, N-ethylmaleinimide, azeleic acid (Th. Schuchardt, Munich). The N-acetoylsulfamoylphenyl-maleinimide was kindly provided by Professor G. Pfleiderer, Frankfurt.

Acyl-CoA Preparations. The synthesis of the acyl-CoA compounds was carried out according to [11]. The radioactive impurity content of the [1-^{14}C] acyl--CoA compounds was determined by thin film chromatography and amounted to 15 to 30%, depending on the CoA charge employed. Thioesters that could not be identified further were involved. Purification was accomplished by means of counter current distribution according to Craig [30 stages, upper phase: petroleum ether-ether (4:5, v/v); lower phase: 0.2 M pyridine, brought to pH 6.1 following addition of glacial acetic acid] or by distribution chromatography using a Sephadex G-50 column. As the buffer for the aqueous phase, we use the above mentioned pyridine-acetic acid buffer with pH = 6.1, while acetic ester was used as the organic phase. The column size was 1.6 × 30 cm for 8 micromoles acyl-CoA.

Following the perculation of the aqueous acyl-CoA solution in the Sephadex column, it was rinsed with a volume of an ester that was 8 times that of the column, after which the acyl-CoA that remained quantitatively in the column was eluded with pyridine-acetic acid buffer. The yield of the acyl-CoA compound obtained by precipitation with perchloric acid was 70%. The impurity content was less than 1% (thin-film chromatographic determination); the phosphate content according to [12]: 3 micromoles PO_4^{-3}/micromoles acyl-CoA. The content determination by measurement of the ultraviolet absorption at 260 millimicrons (adenine band, ε = 16,400 cm^2 $millimoles^{-1}$) and 230 millimicrons (thioester band, ε = 9,400 cm^2 $millimoles^{-1}$) gave corresponding values. In this fashion, the [1^{-14}C] stearoyl-CoA, [1^{-14}C] palmitoyl-CoA and [1^{-14}C] myristoyl-CoA were purified.

Nine-decenic acid was synthesized according to [13]. The resulting product was gas-chromatographically pure.

Radioactively labelled CoASH was prepared according to [14, 15]. Protein determination was carried out according to the Biuret method [16]. ^{14}C-radioactivity was measured in a Packard tricarb scintillation spectrometer, model 314 EX. The scintillation solution contained in one liter of absolute toluol 4 grams of 2,5-diphenyl oxazene and 200 milligrams of 1,4-Bis-(4-methyl-5--phenoxazolyl-) benzene.

The oxidation of the enzymatically produced dicenic acid was performed according to [17].

Thin Film Chromatography. Silica gel G was agitated with water in a ratio of 1:2 and the resulting mixture applied to clean slides with a layer thickness of 0.75-1 millimeter. The plates were activated for 1 hour at a 140°C and stored in a desiccator over $CaCl_2$. After the substances to be separated were applied, chromatography was carried out with an ether-petroleum ether solvent (Kp up to 60°C) -- glacial acetic acid [5:4:1, v/v/v]; running time -- 10 minutes. The silica gel was scratched off the dried plates in several segments into counting dishes and the radioactivity was determined after addition of 15 milliliters scintillation fluid. In this method, the acyl-CoA remained at

the start, while the free fatty acids and the above mentioned thioester-containing impurities migrated with the solvent front.

Isolation of the Microsomal Fraction. In our studies, we used the yeast strain *Saccharomyces cerevisiae* var. ellipsoides. From the culture raised on sloping agar, a large amount of yeast was collected with a platinum loop and placed in 50 milliliters of nutrient solution for culturing (2% bactopeptone difco, 1% yeast extract difco, 2% glucose). After 10 hours of growth at 30°C, the total inoculated solution was transferred to 1 liter of nutrient medium (2 liter Erlenmeyer flask) and cultivated at 30° 12 hours aerobically with agitation. The yeast cell (10 grams), after being centrifuged and washed 3 times with water, were broken down by high speed agitation with 2 parts 0.06 M potassium phosphate buffer, pH 6.5 and 3 parts glass beads for 1 minute. After centrifuging off the coarse cell residue (10 minutes, 10,000 g), the remainder was centrifuged for 30 minutes at 105,000 g. The supernatant fluid was discarded and the precipitate homogenized in 5-8 milliliters of buffer (potassium phosphate, as above) and then centrifuged once more at 105,000 g. Since the particles /50 obtained in this fashion still exhibited a significant fatty acid synthetase activity (determined according to [19]), they were treated in suspension (for 7.5 milligrams protein per milliliter) at 0°C 4 times, 30 seconds each, with ultrasound (Branson sonifier, stage 8, 10 mA). Subsequent centrifuging at 105,000 g for 30 minutes produced a supernatant fluid containing fatty acid synthetase, while the desaturase was contained in the precipitate. The precipitate, after being suspended in a potassium phosphate buffer (0.06 M pH 6.5) was subjected to further centrifuging (59,000 g, 30 minutes), the supernatant fluid discarded and the precipitate placed in a buffer. The particle suspension was divided into several portions and stored at -78°C, and there was no loss of desaturase activity that could be detected within 5 months. Repeated thawing and freezing led to inactivation of the enzyme.

Inhibition Tests. In the test for inhibition of the desaturase system, the enzyme solution was brought to the desired inhibiting material concentration by the addition of a freshly prepared aqueous solution of iodaacetamide, N--ethylmaleinimide and N-acetoylsulfamoylphenyl-maleinimide. Following incubation, whose conditions are described in the tables, the excess SH reagent was removed

by addition of a tenfold quantity of cystein and the inactivation reaction stopped in this fashion; 5 minutes later the remaining activity was measured.

Operation of the Incubation Solution. The solution, stopped with 1 milliliter 40% KOH, was saponified in an autoclave at 1.2 atm, 120°C. Following the addition of 0.5 micromoles of the compound to be determined as the carrier, the solution was acidified with 12 N H_2SO_4 and the fatty acids extracted 3 times with 7 milliliters petroleum ether each time (Kp to 60°C). The markedly reduced petroleum ether solution was mixed with 0.1 milliliters bortrifluoride etherate and 2 milliliter methanol and boiled for 10 minutes with reflux. Following the addition of 2 milliliters of water, the fatty acid methyl ester that was formed was extracted 3 times with 7 milliliters petroleum ether each time and analyzed by means of gas chromatography.

Gas Chromatography. The percentage of desaturation was determined by means of gas chromatography. This was performed with a varian aerograph, model 202-1 B. The fatty acid methyl ester was condensed at the outlet of the thermal conductivity detector and the radioactivity was measured in the scintillation counter. The error in the condensation of the analyzed substance was no more than 5%.

For identification of 9-decenic acid methyl ester and azelaic acid dimethyl ester, a Tricarb combustion furnace (Packard, Frankfurt) was connected to the outlet of the detector with a heatable connecting pipe. The resulting $^{14}CO_2$ was counted in an anthracene throughput cell of the Tricarb scintillation spectrometer model 3101 and recorded with a single channel ratemeter model 280 A with a compensation recorder (Honeywell type 5015).

The analyses were carried out on Chromosorb WAW (80-100 mesh), charged with 11% polyethylene glycolsuccinate. Dimensions of the column: 2 meters × 1/8 inch; furnace temperature for C_{16} and C_{18} fatty acid methyl ester 168°C, C_{14}--fatty acid methyl ester 145°C, C_{10}-fatty acid methyl ester 116°C, azelaic acid dimethyl ester 180°C; gas throughput: 20 milliliters He per minute.

Results and Discussion

Studies of Transferase Activity

In the incubation of unlabelled palimotyl-CoA with radioactive CoASH in the presence of desaturase, radioactive palmitoyl-CoA was formed only in extremely small amounts. The electrophoresis of the solution stopped and concentrated with alcoholic iodine solution for separation of unconverted radioactive CoA (according to [14]) reveals that when equimolar amounts of [^{14}C] CoASH and palimotyl-CoA was added there was no more than 4% exchange in any case. However, it was necessary to note in this regard that the theoretically expected equilibrium value [^{14}C] CoASH-palimotyl-CoA = 1:1 could not be attained, since the enzyme fraction that was employed contained an acyl-CoA hydrolase that hydrolyzed a considerable portion of the palimotyl-CoA during incubation. Under the experimental conditions of the exchange test (250 micrograms enzyme, 18 milligrams per mole palimotyl-CoA, 18 millimicrons per mole [^{14}C] CoASH corresponding to 16 $\times$ 10^3 impulses per minute, 0.18 micromoles glutathiome, 50 micromoles potassium phosphate, pH 6.5, volume 1 milliliter, incubation time 10 minutes at 30°C), 25% of the added substrate was hydrolyzed during incubation. Since 60% of the palimotyl-CoA was oxidized to the unsaturated compound at the same time, as indicated by parallel tests, at least 30% of the radioactivity should have been found in the palimotyl-CoA under the above experimental conditions. This was never the case, so that a conversion of the palimotyl residue from palimotyl-CoA to desaturase as the initial reaction of desaturation can be excluded, at least in yeast cells.

Inhibition Tests With SH Reagents

Ragu et al. [10] described the participation of SH groups in the enzymatic desaturation of saturated fatty acids in rat liver. We performed experiments to inhibit desaturase with iodaacetamide, N-ethylmaleinimide and N-acetoyl-sulfamoylphenyl maleinimide. As we can see from Table 1, in no case could we determine a drop in enzyme activity.

These results indicate that participation of AC-protein component such as has been described in the desaturase system of *Euglena gracilis* [8] and spinach chloroplast [7] can be excluded in the case of yeast. /51

TABLE 1. EXPERIMENTS INVOLVING INHIBITION OF FATTY ACID DESATURASE WITH SH-GROUP BLOCKING REAGENTS

Standard solution: 250 micrograms enzyme, 24.5 millimicron moles [1-^{14}C] palimotyl-CoA corresponding to 6×10^4 impulses per minute, 0.25 micromoles NADPH, 100 micromoles potassium phosphate, pH 6.5, volume 1 milliliter, 10 minutes, start by addition of enzyme (preincubation and preparation, see the experimental section).

Preincubation	Enzymatic conversion				
	Iodaacetamide		N-ethyl-maleinimide	N-acetyl-sulfamoylphenylmaleinimide	
	5 mM	10 mM	0,8 mM	1 mM	4 mM
	Preincubated at				
	25°	25°	0°	30°	20°
min	%	%	%	%	%
0	55	58	40	60	60
2,5	46		37	52	
5	46	34	36		61
10	50	47	39		
20		39			

Studies of Acyl-CoA Hydrolase Activity

Bloomfield and Bloch [1] identified palmitoleoyl-CoA as a product of the enzymatic desaturation of palmitoyl-CoA. However, they were able to determine only a part of the enzymatically converted palmitoyl-CoA as palmitoleoyl-CoA, while the rest occurred as free palmitoleic acid, and attributed this to the activity of an acyl-CoA hydrolase, under whose influence a portion of the thioester was split during the incubation period. Gurr et al. [20] reported that the desaturase system from rat liver is accompanied with very active acyl-CoA hydrolases. By means of our thin film chromatographic method we were able to determine that in a solution without NADPH palmitoyl-CoA and the acyl-CoA compounds which we used are hydrolased by the particle fraction that was used as the desaturase enzyme. The determination of the Michaelis constant of the acyl-CoA hydrolase for various longchain acyl-CoA compounds yielded the values given in Table 2.

TABLE 2. KINETIC CONSTANTS OF THE ACYL-CoA HYDROLASE FOR VARIOUS ACYL-CoA COMPOUNDS

Reaction Solution: 200 micrograms enzyme, 50 micromoles potassium phosphate, pH 6.5, volume 0.5 milliliters, 30°C. Incubation time for stearoyl-CoA 8 minutes, palmitoyl-CoA 10 minutes, myrsitoyl-CoA 5 minutes, lauroyl-CoA 4 minutes. An aliquot of the reaction solution was used directly for thin-chromatographic separation (see the experimental section) at the times given.

Substrate	K_m	V_{max}
	μM	mμMol × min^{-1} × (100 μg enzyme)$^{-1}$
Stearoyl-CoA	22	0,75
Palmitoyl-CoA	24	0,55
Myristoyl-CoA	19	1,18
Lauroyl-CoA	15	2,0

Determination of the Michaelis Constants of the Desaturase

In the determination of the Michaelis constants of the desaturase for stearoyl-CoA, palmitoyl-CoA and myristoyl-CoA, it was necessary to determine that the substrate concentrations were altered rapidly by desaturation and deacylation. Hence, we incubated only for 1.5 minutes so that the change in the substrate concentration during incubation in the least favorable case was no higher than 20%. Figures 1 and 2 show the values plotted according to [21] for the desaturation of palmitoyl-CoA and stearoyl-CoA (curve 1). In Table 3, the values obtained have been summarized and the maximum rates are listed. As indicated by a comparison of Tables 2 and 3, the affinities of the acyl-CoA compound to desaturase were 10 times greater than for acyl-CoA hydrolase.

Product Inhibition of Desaturase

According to [1], the desaturase from yeast is strongly inhibited in its activity by palmitoleic acid in the desaturation of palmitoyl-CoA, but not by oleic acid. At a concentration of 1 millimile palmitoleic acid, the enzymatic conversion of palmitoyl-CoA oxidation under otherwise equal conditions decreases

from 68% to 10%. This finding was monitored by us and could not be concerned with our enzyme preparations. Palmitoleic acid in a concentration of 1 millimole in the incubation solution had no effect on the enzymatic desaturation of palmitoyl-CoA. Likewise, we studied the pair stearoyl-CoA/oleic acid, and here again we were unable to observe any inhibition. The situation was different however when the unsaturated acyl-CoA compounds were added. With these, even at low concentrations, we were able to measure a marked degree of inhibition. It was also found that not only the immediate conversion product of the substrate inhibits the enzyme but also the homologous compound, i.e., in the desaturation of palmitoyl-CoA the addition of oleoyl-CoA also has an inhibiting effect on enzyme activity and conversely the addition of palmitoleoyl-CoA has an effect on desaturation of stearoyl-CoA. These observations agree with the findings of Oshino et al. [22] for desaturase from rat liver, which was likewise markedly inhibited by oleoyl-CoA, but not by free oleic acid.

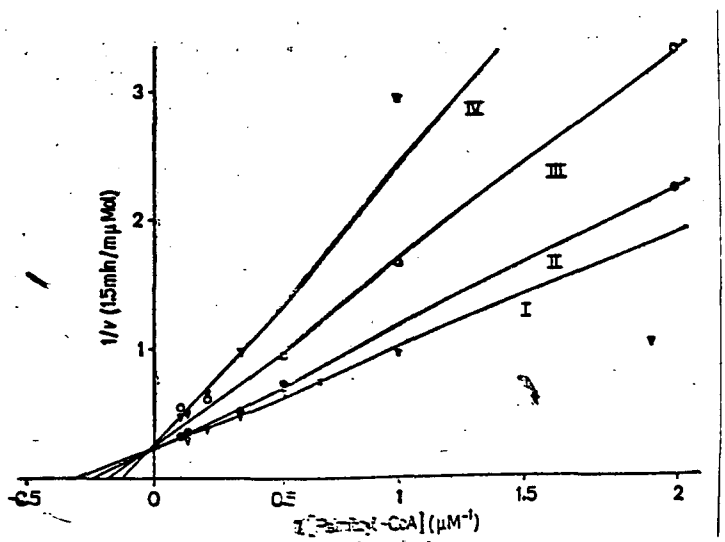

Figure 1. Palmitoleoyl-CoA Inhibits the Desaturation of Palmitoyl-CoA Competitively. Lineweaver-Burk representation [21] of the measurement points. Line 1 -- [blurred] palmitoleoyl-CoA. Lines 2, 3 and 4 with 6, 14 and 20 micromoles palmitoleoyl-CoA (solution, see Table 3).

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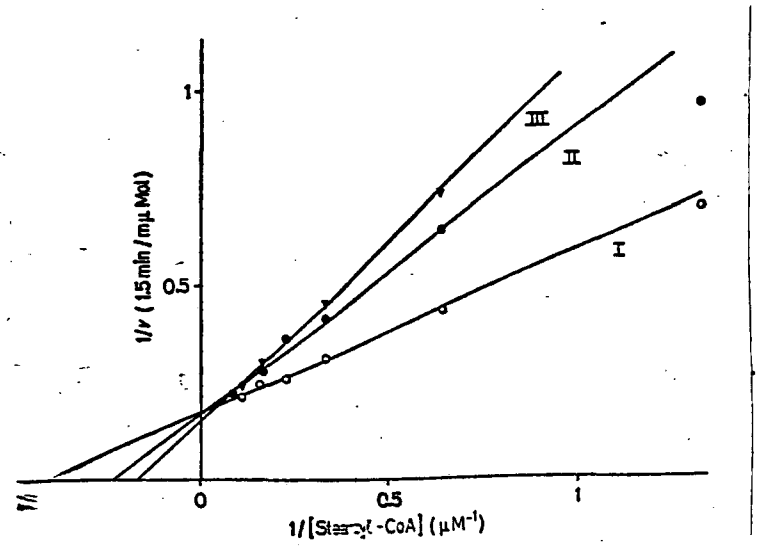

Figure 2. Oleoyl-CoA Inhibits the Desaturation of Stearoyl-CoA Competitively. Measurement point plotted according to the Lineweaver-Burk method [21]. Curve 1: oleoyl-CoA, lines 2 and 3 with 5.6 and 15.2 micromoles oleoyl-CoA (solution, see Table 3).

Substrate	K_m	V_{max}
	μM	mμMol × min⁻¹ × (100μg Enzym)⁻¹
Stearoyl-CoA	2,4	2
Palmitoyl-CoA	3,2	3
Myristoyl-CoA	2,6	2,8

Table 3. KINETIC (blurred) OF FATTY ACID DESATURASE FOR VARIOUS (blurred) COMPOUNDS. Reagent solution: (blurred) enzyme, 1.25 micromoles NADPH, 0.5 millimoles potassium (blurred) pH 6.5, volume 5 milliliters, 30°C, 1.5 minutes, start with (blurred), see the experimental section.

Under the same conditions that were selected for the determination of the [Translator's Note: text blurred] we now study inhibition of the enzyme by palmitoleoyl-CoA. The K_m' values were measured for 3 different concentrations of palmitoleoyl-CoA. Figure 1 shows the values plotted according to [21] (Curves

II-IV). As we can see from these, the enzyme was inhibited competitively by the unsaturated acyl-CoA compound. The inhibitor constant K_i is therefore calculated from [23] and is found to be 2×10^{-5} M.

The procedure was similar for the substrate stearoyl-CoA and the corresponding product oleoyl-CoA (Figure 2, Curves II, III). Here the inhibitor constant K_i was found to be 1.1×10^{-5} M. The discovery that palmitoleic acid and oleic acid in yeast occur in approximately the same amounts [24] and therefore can be explained by the fact that the desaturase has the same substrate specificity for the palmitoyl-CoA and stearoyl-CoA obtained from fatty acid synthetase in equal amounts. The Michaelis constant, maximum rates and inhibition constants /54 are approximately of equal size for the desaturation of palmitoyl-CoA and stearoyl-CoA.

Desaturation of Caprinoyl-CoA

Finally we study the effect of desaturase on [1-^{14}C] caprinoyl-CoA. Figure 3 shows the mass and the corresponding radioactivity chromatogram of such an experiment. We can conclude that the radioactive peak which appears in the gas chromatographic analysis after the capricacidmethylester is identical to the methylester of the 9-decenoic acid. The conversion reaches 30%. In order to determine unambiguously final status of the double bond in the desaturation product, the unsaturated acid was oxidized in a further experiment following addition of 0.5 micromoles of 9-decenoic acid as a carrier to the dicarbonic acid and the resultant azeleic acid was identified by radio gas chromatography following methylation. Figure 4 shows that the radioactive product appears at the site of the authentic azeleic methylester. The 2 smaller radioactive peaks are created by side reactions during ozonization. In control tests with pure 9-decenoic acid we also found small amounts of these compounds that could not be identified further.

Noda et al. [26] reported that 0.1% of the total fatty acids occur as 9--decenoic acid in cow's milk. Dils et al. [27] described the occurrence of this acid in the milk of nursing rats. As far as we know, there has been no mention of the literature of the biosynthesis of this fatty acid. The above investigation makes it clear that this acid can be formed in the same fashion as the other unsaturated fatty acids. In the yeast cell, the above described reaction

possibility plays no role, however, since the capric acid that is formed in intermediary fashion during the buildup of longchain fatty acids is not due to CoA but to the fatty acid synthetase bound to palmitic acid or stearic acid. Hence, the chain length of the saturated and unsaturated fatty acids that occur in yeast is determined simultaneously by fatty acid synthetase. In the yeast cell, caprinoyl-CoA is equally unimportant as a substrate for desaturase as are lauroyl-CoA as well as myristoyl-CoA.

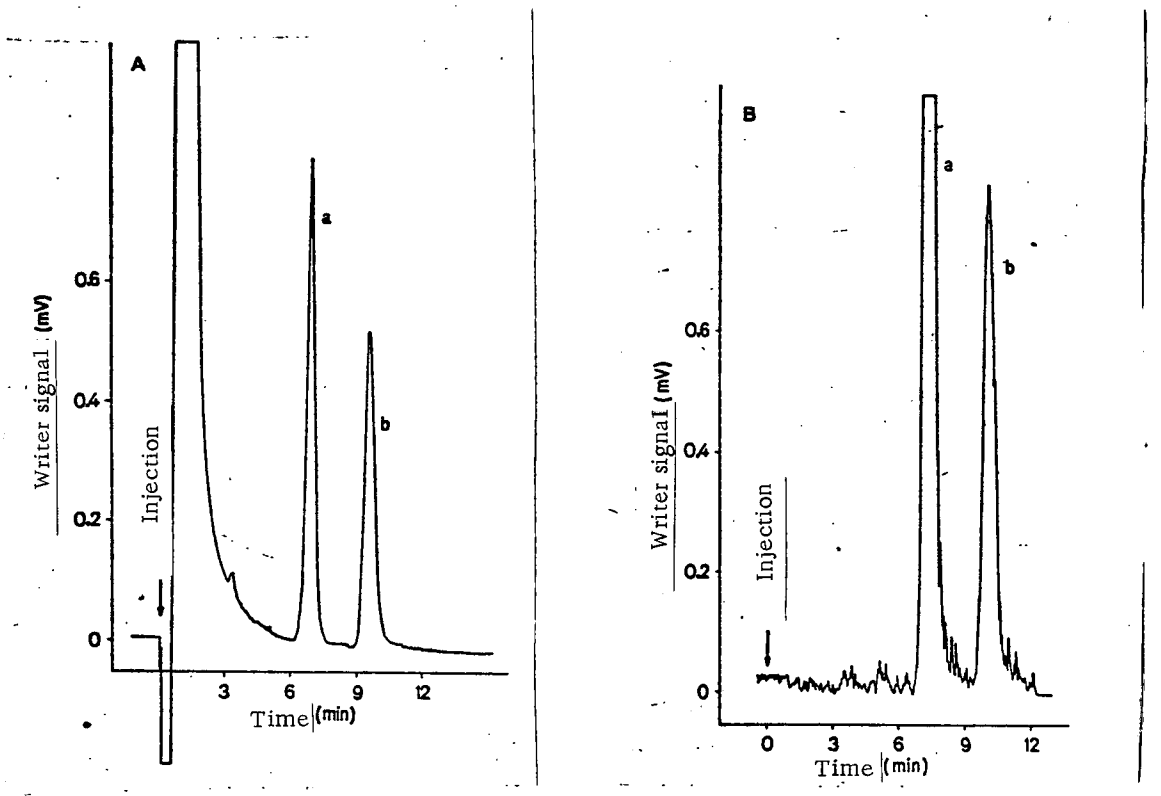

Figure 3. Gas-Chromatographic Separation of Capric Acid In A 9-Decenoic Acid Methylester (A) Mass Gas Chromatogram. (B) radiogas chromatogram, (a) capricacidmethylester, (b) 9-decenoic acid methylester (solution: 1 milligram enzyme, 2.5 micormoles NADPH, 0.25 micromoles [1^{-14} C] caprinoyl--CoA corresponding to 3 x 10^5 impulses per minute, 0.5 millimoles potassium phosphate, pH 6.5, volume 5 milliliters, 30°C, 30 minutes).

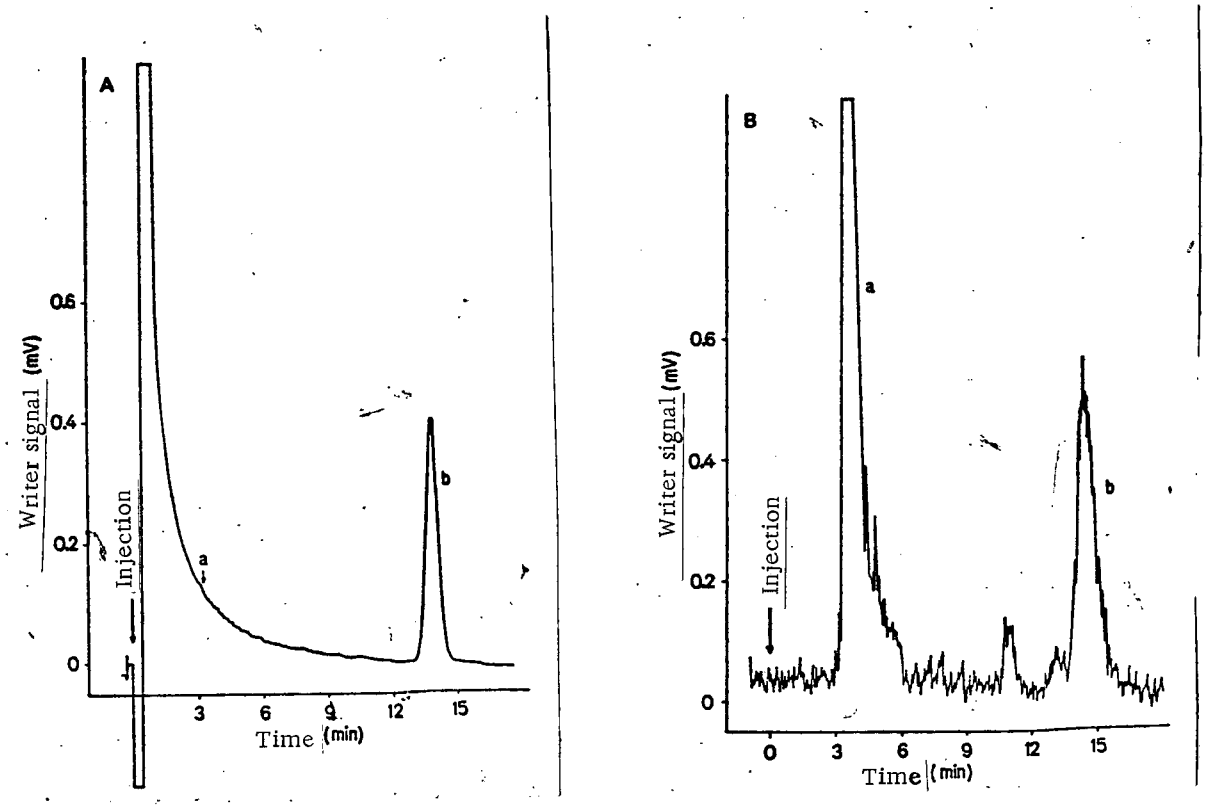

Figure 4. Gas Chromatographic Identification of Azeleic Aciddimethylester. (A) Mass gas chromatogram, (B) radio gas chromatogram, (a) capricacidmethylester, (b) azeleic aciddimethylester (solution, see Figure 3).

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